REMARKS

Claims 1-10 are pending in this application. By this Amendment, claims 1, 9 and 10 are amended. Support for the amendments to claim 1 can be found in the specification as originally filed, for example at page 5, lines 10-12; and in original claim 1. Support for the amendments to claims 9 and 10 can be found in the specification as originally filed, for example at page 6, line 37 - page 7, line 6; and in original claims 9 and 10. No new matter is added by these amendments.

I. Objections to the Specification

The March 30, 2005, Office Action objects to the specification as unclear and asserts that the term "minimal medium" in Table 2 and on page 16 is unclear. In particular, the Office Action asserts that it is not clear whether yeast extract is required for fermentation and that the disclosed minimal medium does not comprise yeast extract. The July 12, 2005, Advisory Action further asserts that the Examiner's points at pages 2 and 3 of the Office Action have not been particularly addressed. Applicant respectfully disagrees.

At page 2 of the Office Action, it is asserted that the example at page 16 of the specification implies that *Bacillus coagulans* is grown on xylose-containing minimal medium, yeast extract is added during fermentation, and xylose is depleted from the medium and converted mostly to lactic acid. The Office Action asserts that these steps occur in this specific order, and that there is no indication of whether the addition of yeast is necessary for growth or fermentation of *B. coagulans*. *See* Office Action, pages 2-3. The Office Action also asserts that Table 2 is unclear because "minimal medium" is used, but the sole example in the specification includes adding yeast extract to minimal medium. *See* Office Action, page 3. Further, the Office Action asserts that the minimal medium defined in the specification is not disclosed as comprising yeast extract. *Id.*

However, minimal medium is a well-known term in the art meaning a medium containing only those nutrients necessary for growth of a bacterium. *See* Declaration of Dr. Arne Olav Sliekers, paragraph 4 (attached). In the instant application, minimal media for *B. smithii* and *B. coagulans* growth are described as including inorganic salts in specified amounts, DAP, DAS, KCl and $MgCl_2$; a buffer, BIS-TRIS; trace elements; pentose carbon sources; and growth factors, biotine, thiamine, methionine, and yeast extract. *See* Specification, page 8, lines 20-22; page 8, line 24 - page 9, line 2. That is, minimal medium for *B. coagulans* growth is defined as including yeast extract, contrary to the Office Action's assertion. Because minimal media are media that contains only the necessary nutrients for *B. coagulans* growth, one of ordinary skill in the art would understand that yeast extract in the minimal medium is necessary for *B. coagulans* growth.

In addition, the example at page 16 of the specification indicates that additional yeast extract is added during fermentation to the minimal medium, which contains already yeast extract, "due to the low biomass concentration achieved after 50 hours of fermentation." *See* Specification, page 16, lines 5-7. That is, the specification clearly indicates that the yeast extract is included in the minimal medium before fermentation as necessary for *B. coagulans* growth and is added during fermentation to increase the biomass of the fermentation broth. *See* Specification, page 8, lines 20-22; page 8, line 24 - page 9, line 2; page 16, lines 5-7; Declaration of Dr. Arne Olav Sliekers, paragraph 4.

Further Applicant respectfully submits that Table 2 is not unclear, because its recitation of minimal medium corresponds to the minimal medium defined at page 8, line 20 - page 9, line 2, which includes yeast extract, as discussed above. This is consistent with the example described in the specification at page 16, in which additional yeast extract is added to the minimal medium during fermentation, due to low biomass concentration. *See*

Specification, page 16, lines 1-13. Thus, the term "minimal medium" and the specification are not unclear.

Accordingly, Applicant respectfully requests withdrawal of the objection to the specification.

II. Claim Rejections Under 35 U.S.C. §112

The Office Action rejects claims 7, 9 and 10 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter that Applicant regards as the invention. Applicant understands from the Advisory Action that the rejection with respect to claim 7 has been withdrawn. Applicant respectfully traverses the rejection with respect to claims 9 and 10.

The Office Action asserts that claims 9 and 10 are confusing because it is unclear whether the "wherein" clause of these claims modifies the purification steps (claims 9 and 10, respectively), the separation step (claim 7) or some undefined step. The Advisory Action indicates that claims 9 and 10 remain rejected under 35 U.S.C. §112, second paragraph, because the "wherein" clauses of claim 9 can "clearly be interpreted as modifying the removing step of claim 9." Applicant respectfully disagrees with the assertions. However, in the interest of furthering prosecution, the wherein clauses of claims 9 and 10 have been amended to clearly set forth that "the moderately thermophilic *Bacillus* species is grown on a chemically defined medium prior to the step of homolactically and anaerobically fermenting." Thus, Applicant respectfully submits that claims 9 and 10 are not indefinite.

Accordingly, reconsideration and withdrawal of the rejections are respectfully requested.

III. Claim Rejections Under 35 U.S.C. §102

The Office Action rejects claims 1-5 and 7 under 35 U.S.C. §102(b) over Payot et al., "Lactic Acid Production by *Bacillus Coagulans* - Kinetic Studies and Optimization of Culture

Medium for Batch and Continuous Fermentations," ENZYME AND MICROBIAL TECHNOLOGY, Vol. 24, 1999, pp.191-199, in light of Godshall et al., "Effect of Macromolecules on Sugar Processing: Comparison of Cane and Beet Macromolecules," AVH ASSOCIATION, 9th Symposium, pp. 23-30. The Advisory Action maintains the rejection and further cites in support of the rejection the Abstracts of Meyer G.A., 1910, Zeitschrift des Vereines der Deutschen Zucker-Industrie 59:1019-1020 and of Pellet H., 1917, Annales de Chimie Analytique et Revue de Chimie Analytique Reunies 22: 43-47. Applicant respectfully traverses this rejection.

The Office Action takes the position that Payot teaches all of the features of claim 1 and dependent claims 2-5 and 7, because Payot teaches producing lactic acid from molasses by *B. coagulans* fermentation. The Office Action asserts that Godshall discloses, in Tables 3 and 4, that molasses comprises glucose, xylose and arabinose. The Advisory Action asserts that Meyer and Pellet teach that molasses contains pentoses.

However, independent claim 1 sets forth a "[p]rocess for preparation of lactic acid and/or lactate, comprising: homolactically and anaerobically fermenting in a fermentation broth a pentose-containing substrate by a moderately thermophilic *Bacillus* species to form lactic acid and/or lactate; wherein the pentose-containing substrate contains a smaller amount of hexose monomers than pentose monomers." Claims 2-5 and 7 depend from claim 1 and incorporate all of the limitations thereof.

Claim 1 sets forth a process comprising a single step, a fermenting step. The "comprising" language of the claim indicates that any process including the claimed fermenting step, with or without other steps, is within the scope of the claim. However, the fermenting step of claim 1 itself is more specific, requiring (a) homolactic and anaerobic conditions, (b) a substrate containing more pentoses than hexoses, and (c) a moderately thermophilic *Bacillus* species. Specifically, claim 1 requires a pentose-containing substrate

that includes more pentose monomers than hexose monomers. None of the cited references teach pentose-containing substrates in which there are more pentose monomers than hexose monomers, as required by the fermentation step of claim 1.

Payot discloses the fermentation of molasses by *B. coagulans* to form lactic acid. *See generally* Payot. However, Payot does not disclose the amounts of pentose or hexose monomers present in molasses. *Id.*

Godshall compares and discloses the compositions of macromolecules of cane and beet sugars, specifically polysaccharides. *See* Godshall, page 26, col. 2, line 5 - page 28, line 26; Tables 3-7. The macromolecules or polysaccharides of cane and beet sugars are made of units including arabinose, xylose and glucose, which are disclosed only as parts of polysaccharides. *Id.* That is, Godshall only discusses these monomer units as parts of the polysaccharides in cane and beet sugars, not as individual monosaccharides. *Id.* In contrast, claim 1 requires a pentose-containing substrate, that is a substrate that contains pentose monomers, not polymerized pentoses. Thus, Godshall teaches substrates containing polysaccharides, but does not teach substrates containing pentose monosaccharides, as required by claim 1. Thus, Godshall does not provide support for the position that Payot's disclosure of molasses fermentation teaches fermentation of pentose-containing substrates that contain smaller amounts of hexose monomers than pentose monomers, as set forth in claim 1.

The Advisory Action further cites Meyer and Pellet for their teachings regarding the composition of molasses, specifically that pentoses are present in molasses. However, neither Meyer nor Pellet teach that molasses is a pentose-containing substrate that contains a smaller amount of hexose monomers than pentose monomers, as set forth in claim 1. Rather, Meyer teaches that, in the beet molasses samples analyzed, pentoses comprised 0.51-0.56 parts, while sucrose, a hexose, comprised 46.7-50.59 parts. *See* Meyer, Abstract. Thus, Meyer

teaches that molasses contains fewer pentose monomers than hexose monomers. That is, Meyer indicates that molasses is not a pentose-containing substrate that contains a smaller amount of hexose monomers than pentose monomers as required by claim 1.

Pellet teaches a method for estimating glucose, a hexose, in molasses. *See* Pellet, Abstract. The amount of glucose is taught to be from 2.6 to 5.6%, but no indication is given for amounts of pentoses present in the molasses. *Id.* Thus, Pellet does not provide support for the position that Payot's disclosure of molasses fermentation teaches fermentation of pentose-containing substrates that contain smaller amounts of hexose monomers than pentose monomers, as set forth in claim 1.

Because molasses is not a pentose-containing substrate that contains a smaller amount of hexose monomers than pentose monomers, as set forth in claim 1, Applicant respectfully submits that claims 1-5 and 7 are patentable over Payot, in light of Godshall, Meyer and Pellet, at least because Payot, even in light of the secondary references, does not disclose fermenting by *B. coagulans* of a pentose-containing substance that contains more pentose monomers than hexose monomers.

For at least these reasons, Applicant respectfully requests reconsideration and withdrawal of the rejection.

IV. Claim Rejections Under 35 U.S.C. §103

A. Claims 1-5 and 7-9

The Office Action rejects claims 1-5 and 7-9 under 35 U.S.C. §103(a) over PCT International Publication No. WO 03/008601 A2 to Green et al. in view of Payot in light of Godshall. The Advisory Action further cites Meyer and Pellet. Applicant respectfully traverses this rejection.

Claim 1 is as set forth above. Claims 2-5 and 7-9 depend, directly or indirectly, from claim 1 and incorporate all of the limitations thereof.

The Office Action cites Green as teaching a process for homolactically fermenting a pentose-containing substance using moderately thermophilic *Bacillus* species, as set forth in claim 1, and teaches the subject matter of the dependent claims. While the Office Action admits that Green does not teach anaerobic fermentation or separation of the biomass or product, it relies on Payot for its teachings on these subjects. Thus, the Office Action takes the position that the subject matter of claims 1-5 and 7-9 would have been obvious over Green, in view of Payot. The Advisory Action asserts that Meyer and Pellet teach that molasses contains pentoses. Applicant respectfully disagrees.

Green discloses thermophilic bacteria, such as those of the *Bacillus* species, for use in converting monosaccharides, such as arabinose, fructose, glucose and xylose, and disaccharides into (L)-lactic acid. *See* Green, page 3, lines 8-11; page 3, lines 22-24; page 4, lines 24-32; page 5, lines 22-23. In particular, Green discloses aerobic assays using *B. smithii* and *B. coagulans* to produce lactate from arabinose, fructose, glucose and xylose. *See* Green, page 7, line 9 - page 10, line 17. However, Green does not disclose or suggest anaerobically fermenting pentose-containing substrates to form lactic acid or lactate, as admitted by the Office Action. The Office Action relies on Payot for such disclosures.

As discussed above, Payot discloses the fermentation of molasses by *B. coagulans* to form lactic acid, but neither of the Payot and Godshall references discloses or suggests the fermentation of pentose-containing substrate that contains a smaller amount of hexose monomers than pentose monomers. *See generally* Payot; Godshall; Meyer, Abstract; Pellet, Abstract. In addition, Payot also does not teach anaerobic fermentation; rather, Payot teaches that biomass is increased with aeration. *See* Payot, page 196, col. 1, lines 13-16.

Because no combination of the cited Green, Payot and Godshall references discloses or suggests at least these features of claim 1, claim 1 and its dependent claims would not have been obvious over Green, Payot and Godshall, individually and in combination.

Applicant respectfully submits that claims 1-5 and 7-9 are patentable over Green in view of Payot and Godshall, at least because no combination of the Green, Payot and Godshall references discloses or suggests anaerobic fermentation by *Bacillus* species of a pentose-containing medium that contains a smaller amount of hexose monomers than pentose monomers. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

B. Claim 6

The Office Action rejects claim 6 under 35 U.S.C. §103(a) over Green in view of Payot, in light of Godshall, and further in view of U.S. Patent No. 4,110,477 to Naruse et al. Applicant respectfully traverses this rejection.

Claim 6 depends from claim 1, which is as set forth above, and further sets forth that the "fermenting is performed by a mixture of moderately thermophilic *Bacillus* species and another lactic-acid producing microorganism." Claim 6 incorporates all of the limitations of claim 1.

The Office Action applies Green, Payot and Godshall in the same manner as to claims 1-5 and 7-9, discussed above, and admits that Green, Payot and Godshall do not disclose or suggest that a mixture of bacteria may be used for fermentation. The Office Action relies on Naruse for its disclosures of fermentation by bacteria mixtures. Thus, the Office Action takes the position that Green, Payot, Godshall and Naruse, in combination, would have rendered the subject matter of claim 6 obvious. Applicant respectfully disagrees.

As discussed above, the combination of Green, Payot and Godshall does not disclose or suggest anaerobic fermentation of pentose-containing substrate in which the pentose-containing substrate contains fewer hexose monomers than pentose monomers. Naruse does not remedy this shortcoming of Green, Payot and Godshall.

Although Naruse does teach mixtures of *Bacillus natto* and lactic acid bacteria for fermentation, Naruse does not teach or suggest either anaerobic fermentation or pentose-containing substrates. *See generally* Naruse. Thus, regardless of its actual teachings, Naruse cannot overcome the deficiencies of the combination of Green, Payot and Godshall. Any combination of the references would still not provide a process that comprises fermentation of a pentose-containing substrate, as claimed.

Applicant respectfully submits that claim 6 is patentable over Green in view of Payot, Godshall and Naruse, at least because no combination of the cited Green, Payot, Godshall and Naruse references discloses or suggests anaerobic fermentation by *Bacillus* species of a pentose-containing medium that contains a smaller amount of hexose monomers than pentose monomers. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

C. **Claim 10**

The Office Action rejects claim 10 under 35 U.S.C. §103(a) over Green in view of Payot in light of Godshall and further in view of U.S. Patent No. 5,002,881 to Van Nispen et al. Applicant respectfully traverses this rejection.

Claim 10 depends from claim 7, which in turn depends from claim 1, set forth above, and further sets forth that the "subjecting the lactic acid and/or lactate to one or more purification steps after separating the lactic acid and/or lactate from the fermentation broth, wherein the moderately thermophilic *Bacillus* species is grown on a chemically defined medium," with claim 7 introducing the separation of lactic acid and/or lactate from the fermentation broth. Claim 10 incorporates all of the limitations of claims 1 and 7.

The Office Action applies Green, Payot and Godshall in the same manner as to claims 1-5 and 7-9, discussed above, and admits that Green, Payot and Godshall do not disclose or suggest purification steps, as set forth in claim 10. The Office Action relies on Van Nispen

for its disclosures of processes for fermenting organic acids in which bacteria are separated from the culture medium and impurities are removed. Thus, the Office Action takes the position that Green, Payot, Godshall and Van Nispen, in combination, would have rendered the subject matter of claim 10 obvious. Applicant respectfully disagrees.

As discussed above, the combination of Green, Payot and Godshall does not disclose or suggest anaerobic fermentation of pentose-containing substrate in which the pentose-containing substrate contains fewer hexose monomers than pentose monomers. Naruse does not remedy this shortcoming of Green, Payot and Godshall.

Although Van Nispen does teach mixtures of *B. coagulans* and lactic acid bacteria for fermentation, Van Nispen does not teach or suggest either anaerobic fermentation or pentose-containing substrates. *See generally* Van Nispen. Thus, Van Nispen, like Green, Payot and Godshall, does not disclose or suggest anaerobic fermentation of pentose-containing substrates. For at least this reason, Van Nispen cannot overcome the deficiencies of the combination of Green, Payot and Godshall. Any combination of the Green, Payot, Godshall and Van Nispen references would still not provide a process that comprises fermentation of a pentose-containing substrate, as claimed.

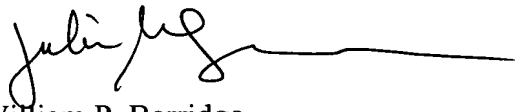
Applicant respectfully submits that claim 10 is patentable over Green in view of Payot, in view of Godshall, and Van Nispen, at least because no combination of the cited Green, Payot, Godshall and Van Nispen references discloses or suggests anaerobic fermentation by *Bacillus* species of a pentose-containing medium that contains a smaller amount of hexose monomers than pentose monomers. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

V. Conclusion

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. Favorable reconsideration and prompt allowance of claims 1-10 are earnestly solicited.

Should the Examiner believe that anything further would be desirable in order to place this application in even better condition for allowance, the Examiner is invited to contact the undersigned at the telephone number set forth below.

Respectfully submitted,



William P. Berridge
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Attachment:
Declaration of Dr. Arne Olav Sliekers

Date: August 30, 2005

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DEPOSIT ACCOUNT USE AUTHORIZATION
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DECLARATION

I, Dr. Arne Olav Sliekers declare the following:

1. I have studied biology at the Rijks Universiteit of Groningen (RUG), the Netherlands. During my education I'm specialized in microbial ecology under supervision of Prof. dr. R.A. Prins, and in microbial physiology at the laboratory of Prof. dr. L. Dijkhuizen. After having received my degree of Master of Science, I have started my PhD study in 1998 at the Technical University of Delft (TU Delft) under supervision of the microbiologists Prof. dr. J.G. Kuenen and Prof. dr. M.S.M. Jetten. The PhD research has resulted to a thesis on the subject of bacteria involved in wastewater treatment. This thesis was published in 2003 and is titled "Ammonia oxidation at the oxic/anoxic interface." I have further published a number of scientific articles in the field of microbiology in several peer-reviewed scientific journals. A list of publications is added as Annex 1.
2. I have read and understood US patent application 10/755,392 in the name of Purac B.V., The Netherlands.
3. The expression "fermentation broth" is a term that is well known to the person of average skill, which in fact belongs to the standard knowledge of any microbiologist. Generally, a fermentation broth is the (liquid) contents of a fermentor wherein bacteria can convert a substrate into one or more products. As an illustration a scientific article using this term, a reference of Adman Hasona et al., Applied and Environmental Microbiology (2002), p. 2651, is added as Annex 2 (see particularly title; abstract, last sentence; Materials and Methods, page 2652, left column; page 2653, Analytical methods, right column; and page 2658, Conclusions, last sentence). I'm of the opinion that the use of the expression "fermentation broth" in US patent application 10/755,392 is

unambiguous and clear to the skilled person, and no further explanation or definition is necessary to understand the meaning of this term.

4. The expression "minimal medium" is a term that is well known to the person of average skill, which in fact belongs to the standard knowledge of any microbiologist. The skilled man knows that a minimal medium is a medium that contains only those nutrients that are necessary for the growth of a bacterium. As an illustration of a scientific article using this term, a reference of Lisa Collins et al., Applied and Environmental Microbiology (1996), p. 848 is added as Annex 3 (see particularly page 848, left column; first sentence of second paragraph; page 850 left column, lines 18, 24, 30, 36, 40 and 44; page 850 right column, lines 1-7). It is my opinion that the use of the expression "minimal medium" in US patent application 10/755,392 is unambiguous and clear to the skilled person, and no further explanation or definition is necessary to understand the meaning of this term.
5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: August, 9, 2005

Signed: 

Annex 1

List of publications

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Schmidt, I., Sliekers, O., Schmid, M., Bock, E., Fuerst, J., Kuenen, J.G., Jetten, M.S.M., Strous, M. (2003) New concepts of microbial treatment processes for the nitrogen removal in wastewater. FEMS Microbiol Rev. 27:481-492

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0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.6.2651–2659.2002

Decreasing the Level of Ethyl Acetate in Ethanolic Fermentation Broths of *Escherichia coli* KO11 by Expression of *Pseudomonas putida estZ* Esterase†

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During the fermentation of sugars to ethanol relatively high levels of an undesirable coproduct, ethyl acetate, are also produced. With ethanologenic *Escherichia coli* strain KO11 as the biocatalyst, the level of ethyl acetate in beer containing 4.8% ethanol was 192 mg liter^{-1}. Although the *E. coli* genome encodes several proteins with esterase activity, neither wild-type strains nor KO11 contained significant ethyl acetate esterase activity. A simple method was developed to rapidly screen bacterial colonies for the presence of esterases which hydrolyze ethyl acetate based on pH change. This method allowed identification of *Pseudomonas putida* NRRL B-18435 as a source of this activity and the cloning of a new esterase gene, *estZ*. Recombinant EstZ esterase was purified to near homogeneity and characterized. It belongs to family IV of lipolytic enzymes and contains the conserved catalytic triad of serine, aspartic acid, and histidine. As expected, this serine esterase was inhibited by phenylmethylsulfonyl fluoride and the histidine reagent diethylpyrocarbonate. The native and subunit molecular weights of the recombinant protein were 36,000, indicating that the enzyme exists as a monomer. By using α-naphthyl acetate as a model substrate, optimal activity was observed at pH 7.5 and 40°C. The K_m and V_{max} for α-naphthyl acetate were 18 μM and 48.1 μmol · min^{-1} · mg of protein^{-1}, respectively. Among the aliphatic esters tested, the highest activity was obtained with propyl acetate (96 μmol · min^{-1} · mg of protein^{-1}), followed by ethyl acetate (66 μmol · min^{-1} · mg of protein^{-1}). Expression of *estZ* in *E. coli* KO11 reduced the concentration of ethyl acetate in fermentation broth (4.8% ethanol) to less than 20 mg liter^{-1}.

In previous studies in our laboratory Ingram et al. replaced the native fermentation pathway in *Escherichia coli* and other enteric bacteria with the homo-ethanol pathway from *Zymomonas mobilis* (18). One of the altered organisms, *E. coli* KO11, has been investigated for commercial use and was shown to ferment the diverse array of sugars present in the polymers of agricultural residues. By using abundant agricultural residues as substrates together with yeast-based fermentation of grain, it may be possible to substantially reduce our dependence on imported petroleum as an automotive fuel (1).

Yeast-based ethanol fermentations result in minor products which copurify with ethanol (5, 6, 11, 26, 30, 38, 41). While many of these products are desirable as organoleptic agents and congeners in beverage alcohols, removal of the contaminating compounds to produce pure ethanol requires additional investment. Ethyl acetate is the most abundant ester produced by yeasts and is particularly difficult to separate from ethanol by distillation (12). This compound has also been found to be a minor product in mixed acid fermentations of many enteric bacteria (*Klebsiella aerogenes*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Enterobacter cloacae*, and *Hafnia alvei*), but it was reported to be absent in *E. coli* fermentations (28). However, a preliminary investigation of distillates obtained from ethanologenic strain KO11 revealed a surprisingly high level of ethyl acetate, in excess of 2 g liter of ethanol^{-1} (Greg Luli, B.C. International, personal communication). The necessity of post-fermentation removal of this contaminant could add to the cost of producing pure ethanol with recombinant *E. coli*.

Ethyl acetate and other esters are produced by alcohol acetyltransferases and are hydrolyzed by esterases (11, 43). The concerted action of these two classes of enzymes determines the levels of ethyl acetate in fermentation broths. Synthesis is thought to result from the transfer of the acetyl moiety from acetyl coenzyme A to ethanol. Potentially, this reaction could be catalyzed by many different acetyltransferases as ethanol accumulates. In yeasts, ethyl acetate production has been ascribed to three acetyltransferases (26), although more than 10 additional candidates are also present on the annotated genome (http://genome-www.stanford.edu/Saccharomyces/). The *E. coli* genome contains at least 13 genes encoding acetyltransferase- or esterase-like proteins with various substrate specificities (4). In ethanologenic strain KO11, production of ethyl acetate during fermentation could result from high ethanol concentrations and a lack of strict substrate specificity. Although it should be possible to reduce ethyl acetate concentrations by eliminating enzymes responsible for ethyl acetate synthesis, these enzymes may also have essential cellular functions. A more efficient, if not more prudent, approach would be to increase the level of esterase with appropriate substrate specificity.

In this paper, we describe a simple method for direct identification of organisms and clones with recombinant DNA that hydrolyze volatile esters by using ethyl acetate as the substrate. This method was used to clone a gene encoding a short-chain aliphatic ester esterase (*estZ*) from *Pseudomonas putida* strain

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† Florida Agricultural Experiment Station Journal Series no. R-08699.

TABLE 1. Bacterial strains and plasmids used in this study

Organism or plasmid	Relevant genotype	Source or reference
Escherichia coli K-12 strains		
AH222	SE2138 *pcnB80 zad-2084*::Tn*10*	This study
BL21(DE3)	*ompT gal dcm lon hsdSB* λDE3	Laboratory collection
DH5α	Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR thi-1 phoA supE44 gyrA96 relA1* ϕ80d*lacZ*Δ*M15*	Laboratory collection
ER1821	e14$^-$ (McrA$^-$) *endA1 supE44 thi-1 relA1*? *rfbD1*? *spoT1*? Δ(*mcrC-mrr*)*114*::IS*10*	New England Biolabs
MRi93	*pcnB80 zad-2084*::Tn*10*	CGSC 7066
SE2138	Prototroph	Laboratory collection
Escherichia coli B	Prototroph	ATCC 11303
Escherichia coli KO11	B − Δ*frd pfl*::pdc_{Zm} $adhB_{Zm}$ *cat*	Laboratory collection
Erwinia chrysanthemi P1	Prototroph	J. Preston
Klebsiella oxytoca M5A1	Prototroph	Laboratory collection
Pseudomonas aeruginosa PAO1	Prototroph	R. Jensen
Pseudomonas putida NRRL B-18435	Prototroph	J. Wolfram
Salmonella enterica serovar Typhimurium LT2	Prototroph	Laboratory collection
Enterococcus sp.	Prototroph	Laboratory collection
Plasmids		
pAH181	pUC18 − *P. putida 'pvdD estZ fpvA 'pvdE*, Ap^r	This study
pAH185	pUC19 − *P. putida 'pvdD estZ 'fpvA*, Ap^r	This study
pAH188	pBR322 − *P. putida estZ*, Ap^r	This study
pAH191	pAH188 − *estZ* (− promoter), Ap^r	This study
pAH199	pAH191 − P_{Zm} (236 bp) − *estZ*, Ap^r	This study
pAH201	pAH191 − P_{Zm} (1.2 kbp) − *estZ*, Ap^r	This study
pAH208	pAH191 − P_{Zm} (147 bp) − *estZ*, Ap^r	This study
pAH213	pAH191 − P_{Zm} (785 bp) − *estZ*, Ap^r	This study
pAH219	pET15b − *estZ*, Ap^r	This study

NRRL B-18435. The encoded protein was purified and characterized. Functional expression of *estZ* in *E. coli* KO11 substantially reduced the level of ethyl acetate in fermentation broth.

MATERIALS AND METHODS

Bacterial cultures. Various derivatives of *E. coli* K-12, *E. coli* B, and other bacteria used in this study are listed in Table 1. Cultures were grown in L broth with appropriate supplements (24). For aerobic growth of nonethanologenic cultures, L broth was used without added sugar. For anaerobic growth, cultures of nonethanologenic strains were supplemented with 0.3% glucose. Ethanologenic strain KO11 (18, 34) was maintained on L agar with xylose (2%). Antibiotics were included in the media at the following concentrations: ampicillin, 100 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; and chloramphenicol, 40 or 600 μg ml^{-1} for KO11 and its derivatives.

Strain AH222, a *pcnB* derivative of wild-type strain SE2138, was constructed by transducing the *pcnB* mutation along with *zad-2084*::Tn*10* from strain MRi93 with phage P1. Tetracycline-resistant transductants were selected, and the presence of the *pcnB* mutation was confirmed by the copy number of plasmid pBR322.

Fermentation of xylose by KO11. Fermentations were carried out in L broth containing 10% xylose as previously described by using 500-ml vessels (29). The cultures were started with an initial cell concentration of 0.33 μg (dry weight) of cells ml^{-1} and were incubated for 48 h. Temperature (35°C), pH (pH 6.5), and agitation (100 rpm) were controlled. Samples were removed at 12-h intervals to measure cell mass, ethanol, and ethyl acetate.

Whole-cell esterase assay (methyl red assay). Esterase activity was determined in whole cells by using methyl red as a pH indicator of the acetate produced by hydrolysis of ethyl acetate. Whatman no. 1 filter paper disks (diameter, 12.5 cm) were soaked in a methyl red solution (1 mg ml^{-1} in 95% ethanol) and allowed to dry. Colonies grown on L agar without added carbohydrate (to prevent acidification) were transferred to the methyl red paper by replica plating and incubated in a desiccator under ethyl acetate vapor. Positive colonies turned red due to the decrease in pH; negative clones appeared yellow on an amber background. With minor modifications, this assay can be used to rapidly screen for esterases which function under different conditions or with different substrates.

Construction of genomic DNA library. Standard methods were used for construction of a genomic library of *P. putida* strain NRRL B-18435 DNA and other DNA manipulations (27). After partial hydrolysis with endonuclease *Sau*3AI, 4.0- to 6.0-kbp fragments of *P. putida* genomic DNA were purified by agarose gel electrophoresis and ligated into a dephosphorylated *Bam*HI site of plasmid vector pUC18. The ligation mixture was transformed into *E. coli* strain DH5α. Plasmid DNA was isolated from the pooled ampicillin-resistant transformants and used as a *P. putida* gene library.

Construction of various esterase plasmids. Additional plasmids were constructed by starting with pAH181 to allow for insertion of alternative promoters. The esterase gene (*estZ*) with flanking DNA was removed as a 2.4-kbp *Sal*I fragment, cloned into vector plasmid pUC19 (plasmid pAH185), and sequenced (GenBank accession no. AY082397). In plasmid pAH185, the *estZ* gene is in the same orientation as the *lac* promoter. The *estZ* gene was removed from pAH185 as a 2.5-kbp *Hin*dIII-*Eco*RI fragment (the *Eco*RI site was filled in by Klenow polymerase) and cloned into the *Hin*dIII and *Ava*I sites (the *Ava*I site was blunt ended by using Klenow polymerase) of pBR322. The resulting plasmid, pAH188, was positive for ethyl acetate hydrolysis as determined by the methyl red assay. A promoter-probe version of pAH188, pAH191, was constructed, in which the region upstream from *estZ* was removed. For this construction, the 5′ 509-bp fragment encoding the N-terminal region of *estZ* was amplified by PCR by using pAH188 as the template, forward primer 5′-AAAAGTCGACGGATCCTAAG GAGTGTGACTTAATGTCCCTGAACCCTGACCTGGCGGCCTA-3′, and reverse primer 5′-CCAGGCTACCACCGACACTGT-3′. The 5′ end of the forward primer included a restriction site for *Sal*I, translational stop codons in all three reading frames, a restriction site for *Bam*HI for promoter DNA insertion, and a Shine-Dalgarno sequence for ribosomal binding. The PCR product was digested with *Sal*I and *Dra*III, and the resulting 342-bp DNA fragment was used to replace the larger *Sal*I and *Dra*III fragment in pAH188. Although *E. coli* strain SE2138(pAH191) was positive for ethyl acetate hydrolase activity, strain AH222(pAH191) carrying a plasmid copy number mutation (*pcnB*) was negative for this hydrolase activity.

Strain AH222(pAH191) was used to clone alternative promoters for *estZ* expression. In these constructs, promoter elements from *Z. mobilis* were used to minimize recombination between plasmid DNA and *E. coli* chromosomal DNA. Endonuclease *Sau*3AI fragments (0.5 to 2.0 kbp) of *Z. mobilis* genomic DNA were used as sources of surrogate promoters. After ligation into the dephosphorylated *Bam*HI site of plasmid pAH191 and transformation of *E. coli* ER1821 (a restriction- and modification-deficient mutant), transformants were pooled for extraction of plasmid DNA. The resulting plasmid mixture was transformed into *E. coli* strain AH222 (*pcnB*), and ampicillin-resistant derivatives were screened

TABLE 2. Effect of EstZ on the fermentation of xylose (10%) by *E. coli* KO11[a]

Organism	Cell mass (g liter^{-1})	Ethanol concn (g liter^{-1})	Ethyl acetate concn (mg liter^{-1})	Esterase activity (U mg of protein^{-1})
KO11	3.43	48	192	0.04
KO11(pAH181)	3.63	48	<20	0.16
KO11(pAH191)	3.52	49	77	0.10
KO11(pAH199)	3.83	51	51	ND[b]
KO11(pAH201)	3.33	51	51	ND
KO11(pAH208)	3.63	49	<20	0.20
KO11(pAH213)	3.52	48	48	ND

[a] Results for completed fermentations (48 h). Esterase activities were measured in cells harvested in the stationary phase (24 h) by using α-naphthyl acetate as the substrate.
[b] ND, not determined.

for ethyl acetate hydrolase activity by using the methyl red assay. The following four clones with the highest activity (rate of color development) were selected for further study: pAH199, pAH201, pAH208, and pAH213.

Purification of EstZ. An *estZ* expression plasmid was constructed to facilitate enzyme purification. The *estZ* coding region was amplified from plasmid pAH181 by PCR performed with forward primer 5'-AAAAAATAAACATATGTCCCT GAACCCTGACCTGGCGG-3' and reverse primer 5'-CTAGTTATTGCTCA GCGCTTCTGATCGCCTGACGTTGA-3'. The forward primer included an *NdeI* site at the predicted ATG start codon. The reverse primer included a *BlpI* site downstream of the translation stop codon (TAA). The PCR-generated fragment was hydrolyzed with *NdeI* and *BlpI*, ligated into the corresponding sites in plasmid pET15b (Novagen), and transformed into *E. coli* strain BL21(DE3) to produce pAH219. With this plasmid, addition of isopropyl-β-D-thiogalactopyranoside (IPTG) induced the production of EstZ as a fusion protein containing an N-terminal histidine tag.

Fresh transformants of *E. coli* strain BL21(DE3) containing pAH219 were grown in 1 liter of L broth in a Fernbach flask with shaking (200 rpm) at 37°C for esterase isolation. When the optical density at 420 nm of the culture reached 0.7 (Spectronic 710; Bausch & Lomb), IPTG was added to a final concentration of 0.1 mM, and the culture was shifted to 23°C. After 4 h of incubation, cells were harvested, washed once with 50 ml of Tris buffer (50 mM Tris-HCl, pH 8.0), resuspended in 25 ml of Tris buffer, and broken by two passages through a French pressure cell at 20,000 lb/in^2. The cell lysate was centrifuged at 30,000 × *g* for 30 min at 4°C, and the supernatant was further clarified by centrifugation at 150,000 × *g* for 60 min at 4°C. The esterase-containing supernatant was loaded on a 5-ml HiTrap affinity chelating column (Pharmacia Biotech) precharged with $NiCl_2$. The column was washed with 50 ml of Tris buffer to remove unbound proteins. Proteins were eluted from the column by using a step gradient of imidazole in Tris buffer (pH 8.0). An imidazole concentration of 20 mM was used to remove nonspecifically bound proteins. EstZ was eluted with 70 mM imidazole in Tris buffer. Fractions containing EstZ were identified on the basis of apparent molecular weight and abundance by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), pooled, and concentrated to 2.5 ml (Centriprep-10; Amicon). This preparation was loaded on a Sephacryl S-200 (Hi-prep 26/60) gel filtration column (Pharmacia Biotech) that had been equilibrated with 50 mM Tris buffer (pH 8.0) containing 150 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 0.5 ml min^{-1}. Fractions containing EstZ were pooled and digested with thrombin (20 U; Pharmacia Biotech) at 16°C for 6 h in the presence of 1 mM $CaCl_2$ to remove the N-terminal His tag. After dialysis overnight against 50 mM Tris buffer at 4°C, EstZ was further purified by using a Q-Sepharose anion-exchange column (15 ml; Pharmacia Biotech) that had been equilibrated with Tris buffer. The esterase was eluted with 0.2 M NaCl, dialyzed overnight in 50 mM Tris buffer (pH 8.0), and stored on ice.

Molecular weight determination. Native molecular weight was determined by gel filtration by using a Sephacryl S-200 gel filtration column (Pharmacia Biotech) in 50 mM Tris buffer (pH 8.0) with 0.15 M NaCl. The molecular weight standards (Sigma Chemical Co.) used were horse heart cytochrome *c* (molecular weight, 12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000), and sweet potato β-amylase (200,000). The subunit molecular weight was determined by SDS-PAGE by using carbonic anhydrase (29,000), ovalbumin (45,000), and bovine serum albumin (66,000) as the standards.

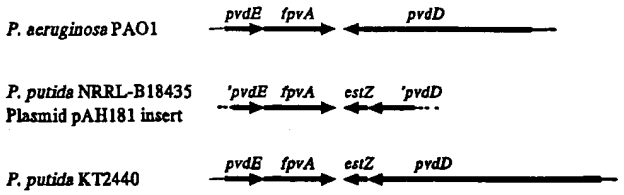

FIG. 1. Comparison of the genetic organizations of the *pvdD estZ fpvA pvdE* gene cluster in *P. putida* NRRL B-18435 (chromosomal fragment in pAH181) and KT2440 (unannotated partial genome from The Institute for Genomic Research) to the genetic organization of the corresponding region in the chromosome of *P. aeruginosa* PAO1 (39).

Enzyme assays. In all enzyme assays, 1 U of esterase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per min. For routine assays, esterase activity was determined in 50 mM sodium phosphate buffer (pH 7.5) by using α-naphthyl acetate as the substrate. An increase in absorbance at 321 nm due to the release of α-naphthol was determined after a 5-min incubation at 37°C. An ε_{321} of 2,740 M^{-1} cm^{-1} was used to calculate the specific activity. In kinetic experiments, the release of α-naphthol was monitored continuously at 37°C by using a water-jacketed cuvette and a Beckman model DU640 spectrophotometer. The cuvette temperature was maintained by using a circulating water bath. Reactions were initiated by adding substrate. Activity was calculated by using the initial rate of product formation. The effects of temperature and pH were also examined by using α-naphthyl acetate as the substrate. For temperature studies, reactants were preequilibrated for 10 min prior to the addition of substrate. For pH studies, Teorell and Stenhagen's citrate-phosphate-borate buffer (50 mM) was used instead of phosphate buffer to allow examination of a wide pH range (3).

Hydrolysis of p-nitrophenyl acetate was measured in 50 mM phosphate buffer (pH 7.5) by determining the increase in absorbance at 400 nm at 37°C due to release of p-nitrophenol (21). An ε_{400} of 16,600 M^{-1} cm^{-1} was used to calculate the enzyme activity.

Esterase activity was also measured by using alcohol esters of acetate as substrates. In these assays, acetate was determined as NADH by using a coupled reaction (acetic acid kit; Boehringer Mannheim, Indianapolis, Ind.). The NADH concentration was determined at 340 nm. The 1-ml reaction mixture (50 mM sodium phosphate buffer [pH 7.5], enzyme, 1 mM substrate) was placed in a 2-ml glass vial and sealed with a serum stopper since many of the substrates are volatile. After 2 to 4 min of incubation at 37°C, the reactions were terminated by incubation at 85°C for 5 min. The acetate present in 0.1 ml of sample was determined by using the acetic acid kit. Under these conditions, the reaction was linear for up to 10 min.

Analytical methods. Ethyl acetate was measured in fermentation broth by gas chromatography (model 3600 chromatograph equipped with a flame ionization detector; Varian, Palo Alto, Calif.) by using a Restek capillary column (30 m by 0.53 mm; Rtx-Volatiles, Bellefonte, Pa.). After injection, the column temperature was held at 50°C for 4 min, raised to 180°C at a rate of 25°C/min, and held at 180°C for 8 min. Dinitrogen was the carrier gas. Under these conditions, the lower limit for measurement of ethyl acetate in broth was approximately 20 mg liter^{-1}. Ethanol was measured as previously described (29).

Materials. Biochemicals were purchased from Sigma Chemical Co. Other organic and inorganic chemicals were obtained from Fisher Scientific and were analytical grade. Restriction endonucleases and DNA-modifying enzymes were purchased from Promega and New England Biolabs. Preliminary sequence data for *P. putida* KT2440 were obtained from The Institute for Genomic Research website (http://www.tigr.org).

RESULTS AND DISCUSSION

The problem: ethyl acetate production by ethanologenic strain KO11. In a typical fermentation containing 10% xylose, ethanologenic *E. coli* strain KO11 produced about 4.8% ethanol and 192 mg of ethyl acetate liter^{-1} (Table 2). This level of ethyl acetate is 4- to 10-fold higher than the level typically observed with yeasts (5, 6, 11, 26, 30, 38, 41). In yeasts and presumably other organisms, the level of ethyl acetate is mod-

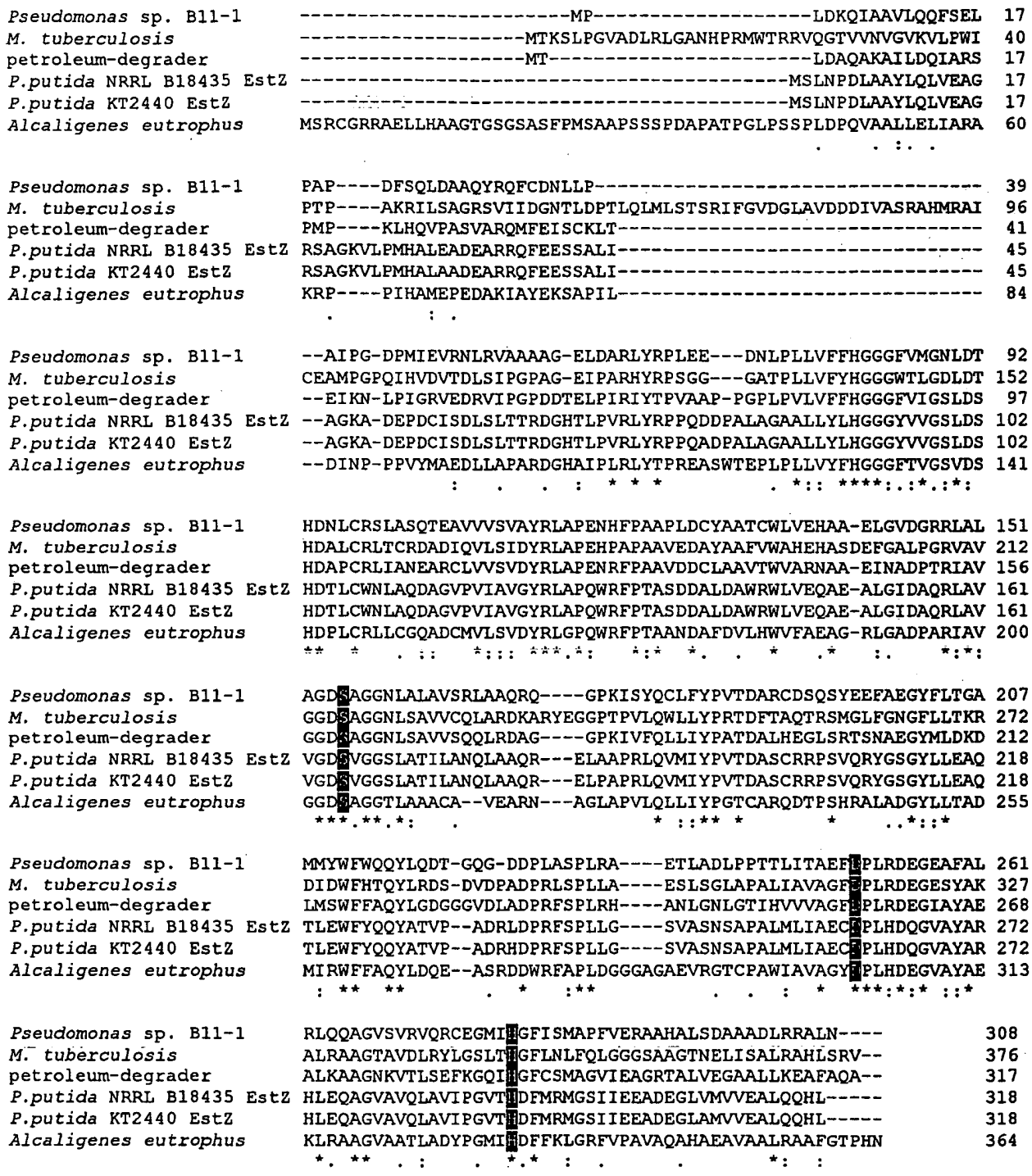

FIG. 2. Comparison of the translated sequence of *P. putida* NRRL B-18435 EstZ with sequences of other family IV lipases/esterases (7, 8, 33, 42; www.tigr.org). The sequence of an esterase from an unidentified, petroleum-degrading bacterium, strain HD-1 (33), is also included. Amino acids that are conserved in all six protein sequences are indicated by asterisks below the sequences. Amino acids which are similar are indicated by colons, and functionally compatible amino acids are indicated by dots. Amino acids in the proposed catalytic triad are highlighted.

ulated by the activities of alcohol acetyltransferases and esterases (11). This compound has no known physiological function in yeasts. The higher level of this compound in fermentations with ethanologenic *E. coli* KO11 is presumed to result from either higher acetyltransferase activities which synthesize ethyl acetate or a lack of esterases which hydrolyze ethyl acetate. When tests were performed by using the methyl red assay (whole cells), *E. coli* B, KO11, and K-12 wild-type strains

lacked the ability to hydrolyze ethyl acetate and failed to utilize ethyl acetate as a sole carbon source for growth. A variety of other bacteria (*Klebsiella oxytoca* strain M5A1, *Salmonella enterica* serovar Typhimurium strain LT2, *Erwinia chrysanthemi* strain P1, *Pseudomonas aeruginosa* strain PAO1, and *Enterococcus* spp.) were tested by using the methyl red assay and were found to lack the ability to hydrolyze ethyl acetate. Of the organisms tested, only *P. putida* NRRL B-18435 produced high levels of this activity.

Cloning the gene responsible for ethyl acetate hydrolysis. *E. coli* strain SE2138 was transformed with a gene library from *P. putida* NRRL B-18435. Approximately 10,000 transformants were screened for ethyl acetate hydrolase activity by using the methyl red assay. Clones which turned bright red within 5 min of exposure to ethyl acetate vapor were selected from a replica plate and retested. Of the 20 positive clones, 1 clone expressing the highest activity (rate of color development) was selected for further study, and the plasmid was designated pAH181.

Plasmid pAH181 (Fig. 1) contained a 6.7-kbp insert with four open reading frames (ORFs). These ORFs were identified by comparison to other known genes by using Blast (www.ncbi.nlm.nih.gov). One of the ORFs contained the C-terminal 594 amino acids of a siderophore synthase (nonribosomal peptide synthesis) resembling PvdD in *P. aeruginosa* PAO1 (32) and *P. putida* WCS358 (GenBank accession no. CAC32046), with 61 and 62% identity, respectively. This C-terminal segment contained the thioesterase motif found in peptide synthetases associated with nonribosomal synthesis of peptide antibiotics (15, 25). The DNA region containing the *pvdD*-like gene was also localized on the incomplete, unannotated genome sequence of *P. putida* strain KT2440 (www.tigr.org). From this database, the full-length gene in *P. putida* NRRL B-18435 appears to encode a 3,089-amino-acid protein.

The ORF immediately following *pvdD* encodes an esterase gene and was designated *estZ*. The translated *estZ* protein (318 amino acids) is similar to family IV esterases/lipases and to a hormone-sensitive lipase (Fig. 2) (2, 7, 23). The levels of amino acid sequence identity between EstZ and the other esterases/lipases listed in Fig. 2 varied between 30 and 41% (7, 8, 33, 42). The consensus motif for esterases, LAVVGDSVGG (10, 17), is located between positions 159 and 168 of the translated EstZ sequence. The amino acid serine at position 165 corresponds to the active site serine. EstZ also contains aspartate and histidine in the conserved region, completing a proposed catalytic triad (16, 35). Consistent with the lack of a signal sequence for translocation, EstZ activity was found in the cytoplasm of both *P. putida* NRRL B-18435 and recombinant *E. coli* (data not shown).

The *estZ* gene is located immediately downstream from the *pvdD* gene on the unannotated genome of *P. putida* KT2440 also (www.tigr.org). Both genes appear to be in the same operon, part of a pyoverdine synthesis gene cluster in *P. putida*. The predicted amino acid sequence of EstZ from strain KT2440 was 98% identical to the sequence of the cloned *estZ* gene product from strain NRRL-B18435. The five amino acids which differ reside in the nonconserved regions of family IV esterases.

The other two ORFs (pAH181) appear to be transcribed in the opposite direction from *pvdD* and *estZ* (Fig. 1). The third ORF (*fpvA*) was identified as a ferric pyoverdine receptor protein (39% identity with FpvA from *P. aeruginosa* PAO1) (32). Based on protein sequence similarity, the fourth ORF (*pvdE*) appears to encode the C-terminal portion of an ATP-dependent ABC membrane transporter associated with pyoverdine biosynthesis (66% identity with PvdE in *P. aeruginosa* PAO1) (31).

TABLE 3. Kinetic properties of the *P. putida* esterase

Substrate	K_m (μM)	V_{max} (U mg of protein^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
α-Naphthyl acetate	18	48	29	1.6
ρ-Nitrophenyl acetate	54	106	64	1.2

The ethyl acetate esterase activity encoded by plasmid pAH181 could be provided by either incomplete PvdD with the thioesterase motif or EstZ (Fig. 1). It is interesting that the genome of a related organism, *P. aeruginosa* PAO1, lacks the *estZ* gene between the homologous *pvdD* and *fpvA* genes (39). *P. aeruginosa* PAO1 failed to hydrolyze ethyl acetate, which is consistent with the hypothesis that *estZ* is the source of ethyl acetate esterase activity. To confirm this, a DNA fragment containing only the *estZ* gene was cloned into plasmid vector pBR322 to produce plasmid pAH188. Recombinant *E. coli* strains harboring pAH188 were ethyl acetate hydrolase positive. Although EstZ hydrolyzed ethyl acetate, the physiological role of this esterase in *P. putida* is apparently in pyoverdine biosynthesis. The specific role of EstZ in this process and its in vivo substrate have not been identified.

In *E. coli*, the *aes* gene encodes an enzyme related to family IV esterases (19, 36) which exhibits 23% identity with the translated EstZ from *P. putida* NRRL B-18435. The Aes enzyme has been shown to hydrolyze valerate esters preferentially over acetate as the acid moiety (19). The failure of native *E. coli* to hydrolyze ethyl acetate could be due to inadequate levels of Aes. In gene array studies, the mRNA levels for *aes* were found to be low (40).

Biochemical properties of EstZ. Recombinant EstZ was purified 35-fold almost to homogeneity based on SDS-PAGE (data not shown). Both the native molecular weight (as determined by gel filtration) and the subunit molecular weight (as determined by SDS-PAGE) were approximately 36,000, indicating that EstZ functions as a monomer. The specific activities of purified EstZ were 42 and 66 U mg of protein^{-1} for α-naphthyl acetate and ethyl acetate, respectively. Kinetic properties of the EstZ were determined by using two chromogenic substrates, α-naphthyl acetate and ρ-nitrophenyl acetate; the highly volatile nature of ethyl acetate hindered kinetic studies with this substrate (Table 3). Both chromogenic substrates were hydrolyzed with Michalean kinetics. The affinity for ρ-nitrophenyl acetate was threefold lower than that for α-naphthyl acetate. However, the V_{max} with ρ-nitrophenyl acetate was approximately twice that with α-nitrophenyl acetate. Thus, the higher turnover number obtained with ρ-nitrophenyl acetate was compensated for by the lower affinity for this substrate.

The effects of pH and temperature on EstZ activity were determined by using α-naphthyl acetate as the substrate. This enzyme was active between pH 6.0 and 10.5, with maximum activity at pH 7.5 (Fig. 3). Increasing the assay pH above this value led to a linear decrease in specific activity. EstZ retained

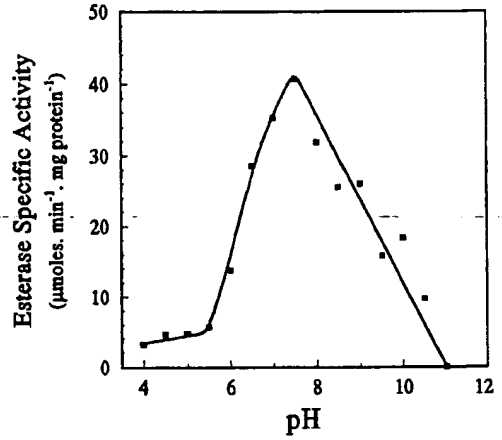

FIG. 3. Effect of pH on EstZ activity. α-Naphthyl acetate was used as the substrate.

40% of the maximal activity at pH 10.0 but was inactive at pH 11.0. In this regard, EstZ appears to be similar to the lipase from *Pseudomonas* sp. strain B11-1 (7) and to an esterase from *Streptomyces diastatochromogenes,* (20) but differs from a *Pseudomonas fluorescens* esterase which retained 80% of the maximal (pH 7.5) activity at pH 10.0 (22). At pH 5.5, EstZ was only minimally active (about 10% of the maximal activity). The increase in activity between pH 5.5 and 7.5 with half-maximal activity near pH 6.5 suggests that protonation of a histidine residue is essential for optimal activity (histidine pK_a, 6.3). A histidine at position 291 is conserved in all family IV esterases and has been implicated in the catalytic triad of the hormone-sensitive lipase (2), an ortholog of the *P. putida* EstZ esterase.

EstZ esterase was optimally active at temperatures between 35 and 45°C, with maximal activity near 40°C (Fig. 4). The energy of activation was calculated to be 17.4 kcal mol^{-1}. This low activation energy is similar to the value of 11.2 kcal mol^{-1} obtained with the lipase from *Pseudomonas* sp. strain B11-1 (7).

Substrate specificity of EstZ. The activity of EstZ decreased with increasing chain length of the acid moiety (Table 4). It is interesting that this esterase failed to hydrolyze α-naphthyl butyrate, although α-naphthyl caproate was hydrolyzed. Esters with chain lengths of eight carbons or more were not hydrolyzed. A similar decline in activity with increasing chain length of the aliphatic acid was observed for the lipase from *Pseudomonas* sp. strain B11-1 (7) and for an esterase from *P. fluorescens* (22). Although α-naphthyl acetate was hydrolyzed by EstZ with a maximum specific activity of 42 U mg of $protein^{-1}$, β-naphthyl acetate was a poor substrate (about 20% of the activity with α-naphthyl acetate). Other aromatic substrates, such as ρ-nitrophenyl acetate, were also hydrolyzed, but at a lower rate, by EstZ (Table 4).

TABLE 4. Effect of chain length of the acid moiety on EstZ activity

Substrate	Esterase activity (U mg of $protein^{-1}$)
α-Naphthyl acetate	42
α-Naphthyl propionate	33
α-Naphthyl butyrate	<1
α-Naphthyl caproate	15
α-Naphthyl caprylate	<1
α-Naphthyl laurate	<1
β-Naphthyl acetate	9
ρ-Nitrophenyl acetate	11

A series of aliphatic esters of acetate were also tested as substrates for EstZ (Fig. 5). Increasing the chain length of the alcohol moiety from one carbon to three carbons increased the hydrolysis rate of the ester (Fig. 5). The highest specific activity was observed with propyl acetate as the substrate (96 U mg of $protein^{-1}$). Increasing the chain length to more than three carbons led to a rapid decline in activity. With decyl acetate, the EstZ activity was less than 5% of that with propyl acetate. These results show that the preferred substrate for this enzyme is propyl acetate. Although EstZ hydrolyzed aromatic esters like naphthyl acetate and nitrophenyl acetate, higher activities were obtained with water-soluble aliphatic short-chain esters. This enzyme was sensitive to dimethyl sulfoxide (DMSO), which is consistent with a preference for aliphatic esters (Fig. 6). In the presence of 30% DMSO, the enzyme activity was less than 10% of the aqueous value. In this regard, EstZ is different from the related lipase from *Pseudomonas* sp. strain B11-1, whose activity was enhanced by DMSO (7).

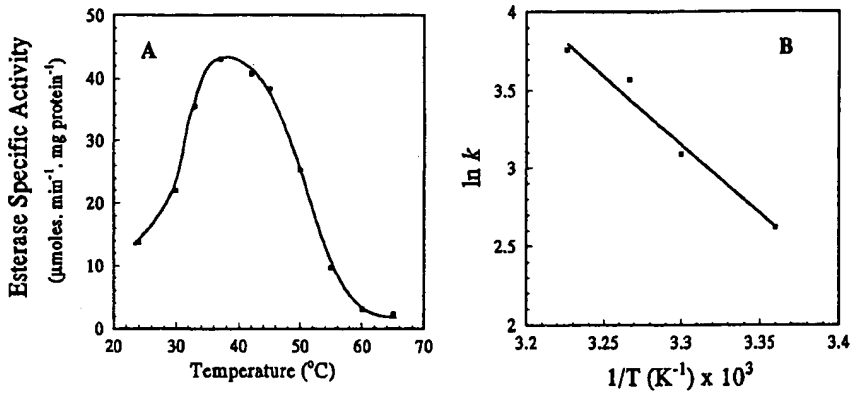

FIG. 4. Effect of temperature on EstZ activity. α-Naphthyl acetate was used as the substrate. (A) Temperature plot; (B) Arrhenius plot.

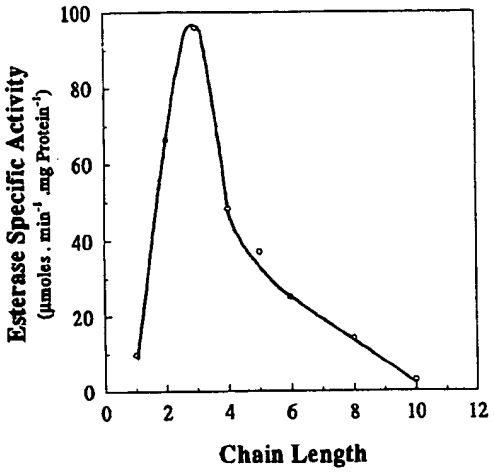

FIG. 5. Effect of aliphatic alcohol chain length on EstZ activity, determined by using acetate esters as substrates.

Effect of inhibitors on EstZ activity. All esterases and lipases, including the hormone-sensitive lipase, contain the α/β hydrolase fold and a catalytic triad with serine (nucleophile), a catalytic acid residue (asparate or glutamate), and histidine (35). EstZ from *P. putida* is also expected to conform to this structure based on the conservation of the amino acid sequence (Fig. 2). By using the crystal structure of a thermophilic esterase from *Alicyclobacillus acidocaldarius* as the model (9), a tertiary structure for EstZ was computed with SwissModel (13, 14, 37). The predicted structure exhibited an α/β hydrolase fold and close proximity of the conserved amino acids in the catalytic triad for EstZ. Requirements for serine and histidine for catalytic activity were confirmed by using specific inhibitors (Table 5). The serine protease inhibitor phenylmethylsulfonyl fluoride completely inhibited the EstZ activity, while a similar concentration of eserine resulted in 75% inhibition. Differences in the structure of the aromatic rings between the two inhibitors may influence the level of inhibition. Inhibition by diethylpyrocarbonate provided further evidence that a histidine residue is important for activity. EstZ has four cysteines, one of which (cysteine 107) is conserved in the six family IV esterases listed in Fig. 2. Two sulfhydryl reagents, mercuric chloride and naphthol AS-D chloroacetate, inhibited EstZ activity, suggesting that one or more of the cysteines have an important role. A lack of inhibition by EDTA indicates that a divalent cation is not essential for activity. These inhibitor studies and predicted models suggest that the general structure and reaction mechanism of EstZ from *P. putida* are similar to the general structure and reaction mechanism of other esterases.

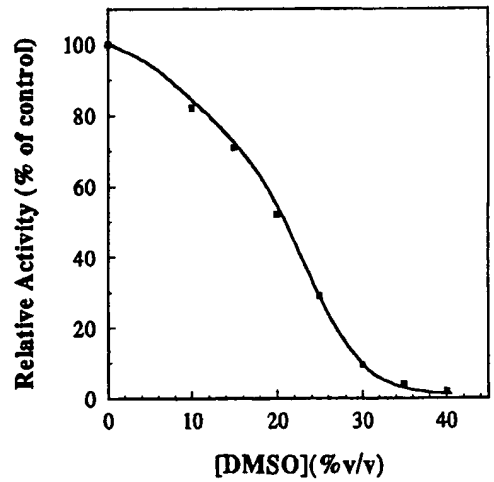

FIG. 6. Effect of DMSO on EstZ activity. 4-Methylumbelliferyl acetate was used as the substrate. The specific activity without DMSO was 12.6 U mg of protein^{-1}.

TABLE 5. Effects of inhibitors on EstZ activity

Compound	Esterase activity (U mg of protein^{-1})
None	40
Phenylmethylsulfonyl fluoride	<1
Eserine	11
Mercuric chloride	2
Naphthol AS-D chloroacetate	<1
Diethylpyrocarbonate	<1
EDTA	31

Utility of *estZ*: effect of *E. coli* KO11 on the level of ethyl acetate in ethanolic beers. The EstZ esterase from *P. putida* exhibited a preference for short-chain aliphatic esters and may be useful for reduction of ethyl acetate in fermentation broths. To examine this possibility, KO11 was transformed with pAH181 containing the 6.7-kb fragment of DNA from *P. putida* NRRL B-18435 which contains *estZ* (Fig. 1). Expression of *estZ* in KO11 was confirmed by using α-naphthyl acetate as a substrate (Table 2). Native esterases in KO11 also hydrolyzed α-naphthyl acetate but had negligible activity with ethyl acetate. Based on studies performed with highly purified recombinant enzyme, the specific activity of EstZ with α-naphthyl acetate was found to be approximately 0.66 that with ethyl acetate (Table 4 and Fig. 5). Based on this ratio and the difference in esterase activity (α-naphthyl acetate hydrolysis) between KO11 and KO11(pAH181), the hydrolytic activity for ethyl acetate in KO11(pAH181) was estimated to be 0.18 U mg of cell protein^{-1}.

Fermentations with 10% xylose were completed within 48 h, producing more than 95% of the maximum theoretical yield for ethanol (0.51 g of ethanol per g of xylose) (Table 2). Although KO11(pAH181) and unmodified KO11 produced similar cell densities (3.63 and 3.43 g liter^{-1}, respectively) and similar levels of ethanol (4.8%), the concentration of ethyl acetate in the KO11(pAH181) beer (<20 mg liter^{-1}) was at least ninefold lower than that in the beer produced with unmodified KO11 (Table 2). These results clearly establish the utility of recombinant EstZ as an effective way to reduce ethyl acetate concentrations in ethanolic beers produced with recombinant *E. coli*.

Additional plasmid constructs were made with *estZ* to introduce promoter elements since in plasmid pAH181 *estZ* is apparently transcribed from the *lacZ* promoter in the vector. These constructs included a regulated T7-based expression

vector (pAH219) which can be induced with IPTG to produce high levels of EstZ for enzyme purification or as a reagent for the treatment of beers, as well as pBR322-based vectors for expression in ethanologenic *E. coli*. The results obtained with five pBR322-based constructs are shown in Table 2. Plasmid pAH191 contains only the *estZ* coding region (with a Shine-Dalgarno sequence) preceded by a unique *Bam*HI site. Expression of *estZ* in this plasmid is presumed to be from the upstream *tet* promoter or promoter-like DNA sequence in the vector, plasmid pBR322. Plasmids pAH199, pAH201, pAH208, and pAH213 are derivatives of pAH191 which contain different fragments of *Z. mobilis* DNA that serve as promoters in *E. coli*. When transformed into KO11, all of these pBR322-based plasmids expressed ethyl acetate-hydrolyzing activity (as determined by the methyl red assay) and reduced the level of ethyl acetate present in beers to less than one-third the level produced by unmodified KO11 (Table 2). Beer produced with the most effective pBR322-based construct, KO11(pAH208), contained less than 20 mg of ethyl acetate $liter^{-1}$. Cell growth and ethanol yield were not affected by inclusion of these plasmids.

Conclusions. These studies showed that a genetic approach can be used to effectively reduce the level of ethyl acetate, an undesired coproduct, in ethanol beers produced with *E. coli* KO11. The genetic improvement, expression of plasmid-borne *estZ* from *P. putida* in KO11, was accomplished without decreasing the effectiveness of the biocatalyst. Cloning of *estZ* was facilitated by a simple screening method, minor modifications of which should prove to be generally useful for isolation of additional esterases with alternative reaction optima and substrates. Native or recombinant EstZ can be used to decrease ethyl acetate levels in fermentation broths as a postfermentation treatment or as an in vivo recombinant enzyme produced by the biocatalyst.

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Development of a Defined Minimal Medium for the Growth of *Edwardsiella ictaluri*†

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In this report, a complete defined medium and a minimally defined medium are described for *Edwardsiella ictaluri*. The complete defined medium consists of 46 individual components, including a basal salt solution, glucose, magnesium sulfate, iron sulfate, six trace metals, four nucleotides, 10 vitamins, and 19 amino acids. This medium supports growth in broth and on solid media. Optimal growth at 30°C was obtained at pH 7.0, and at an osmolality of 390 mosmol/kg of H_2O, with a glucose concentration of 4 g/liter. The defined minimal medium reduces the 46 components of the complete medium to eight essential components, including the basal salt solution, glucose, magnesium sulfate, pantothenic acid, and niacinamide. In addition, specific amino acids that depend on the specific requirements of the individual strains of *E. ictaluri* are added.

Edwardsiella ictaluri was first isolated from diseased channel catfish in 1976 and is the causative agent of enteric septicemia of catfish, the most serious disease affecting commercial catfish production (7). The organism is a weakly motile, gram-negative rod with peritrichous flagella and is cytochrome oxidase, indole, and H_2S negative (8, 17). Growth is relatively slow and generally requires a rich, complex medium. Although a selective medium for the isolation of *E. ictaluri* has been described previously (21), no work has been conducted to define the nutritional requirements for this important pathogen of farm-raised catfish.

Complete defined media contain complex mixtures of bacterial nutrients, while minimal defined media contain only those nutrients essential to the growth of a given species. These media have been used for the examination of microbial physiology (11), nutrition (12, 20), elicitation and accumulation of toxins (15), determination of growth requirements, and the development and characterization of auxotrophic mutants (6, 9). In addition, because the expression of virulence factors is often regulated by environmental conditions (13), defined media have been used to examine nutritional control of the expression of bacterial virulence mechanisms (2, 3, 19). At present, major virulence factors and pathogenic mechanisms for *E. ictaluri* have not been identified, possibly because of the current dependence on complex media for propagation of the organism. The development of a defined minimal medium that supports the growth of *E. ictaluri* is essential to the design of reproducible studies relating to biochemical, physiological, and genetic variation within the species and to subsequent studies on virulence and pathogenesis. This study was undertaken to determine the minimal nutritional requirements of *E. ictaluri* by first developing a complete defined medium, from which minimal requirements could be determined, ultimately leading to the development of a minimal defined medium that supports growth of *E. ictaluri*.

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MATERIALS AND METHODS

Bacterial strains. All *E. ictaluri* strains in this study were originally isolated from moribund channel catfish. Strains 89-9, 90-476, 91-581, 91-638, 92-266, 93-146, 93-154, 93-170, 93-264, and 93-297 were isolated at the Aquatic Animal Diagnostic Laboratory, Louisiana State University School of Veterinary Medicine, strains 587-671 and 587-673 were isolated at the Mississippi State Cooperative Extension Service Diagnostic Lab in Stoneville, strain 589-521 was isolated at the South Carolina Aquatic Diagnostic Lab, Clemson, and strain 83-189 was isolated at the Auburn University Diagnostic Lab, Auburn, Ala. Stock cultures were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) in a rotary shaker at 30°C and stored frozen in BHI broth with 20% glycerol at −70°C.

Chemicals and growth media. All chemicals were of analytical grade unless stated otherwise. Amino acids, vitamins, purines and pyrimidines, sugars, and inorganic salts were purchased in the highest grade available from Sigma Chemical Co. (St. Louis, Mo.). Sodium chloride, dibasic sodium phosphate, ammonium chloride, and glucose were purchased from EM Sciences (Gibbstown, N.J.). Calcium chloride and monobasic potassium phosphate were obtained from Mallinckrodt (Chesterfield, Mo.).

Preparation of glassware. Glassware was soaked in a 5% (vol/vol) solution of Roccal II (National Laboratories, Montvale, N.J.) overnight, rinsed once in distilled water, immersed overnight in 10% nitric acid, rinsed six times in distilled water, dried in an oven at 60°C, and autoclaved.

Growth conditions and assessment of growth. Starter cultures were inoculated from single colony isolates grown from frozen BHI broth-glycerol stock cultures into glass test tubes containing 5 ml of complete defined medium (see recipe below) and grown overnight at 30°C on a Cel-Grow tissue culture rotator (Lab-Line Instruments, Inc., Melrose Park, Ill.). Starter cultures for optimization of osmolality, pH, carbon source, and carbon concentration were inoculated at a 1:50 dilution into 50 ml of test medium in 250-ml Klett flasks (Kontes, Vineland, N.J.), incubated in a rotary shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 30°C, and assayed for growth on a Klett-Summerson model 800-3 photoelectric colorimeter with a green filter (Klett Manufacturing Co., Inc., Long Island, N.Y.). Starter cultures for minimalization of minerals, vitamins, and amino acids were inoculated at a 1:50 dilution into 5 ml of test medium in glass test tubes, grown at 30°C on a Cel-Grow tissue culture rotator, and assayed for growth as described above. To ensure elimination of carryover nutrients, cultures grown in complete defined medium were passaged twice at 24-h intervals, while cultures grown in minimal defined medium were passaged at 48-h intervals.

Defined medium optimization. Preliminary work in our lab indicated that M9 salts (18) supplemented with glucose and Casamino Acids (Difco) supported growth of *E. ictaluri*. Subsequent replacement of the Casamino Acids with vitamins, purines and pyrimidines, trace minerals, and amino acids resulted in a complete defined medium that was used to optimize osmolality, carbon source and concentration, and pH as described below. Water for all media was deionized and double distilled prior to use. Stock solutions were prepared as described in Table 1. Stock solutions were filter sterilized individually with a 0.2-μm-pore-size filter and stored at 20°C for not more than 30 days. Medium components were prepared separately as the following: (i) basal salts, (ii) carbohydrates, (iii) minerals, (iv) vitamins, (v) purines and pyrimidines, and (vi) amino acids. Data for the complete defined medium optimization are the means of triplicate cultures repeated twice and recorded at 12 and 24 h.

Optimal osmolality was determined in Klett flasks containing 50 ml of com-

TABLE 2. Effect of osmolality on the growth of *E. ictaluri* in complete defined medium

mosmol/kg of H_2O	Klett units[a]	
	12 h	24 h
120	229 ± 16.8B	235 ± 11.3C
260	363 ± 3.8A	383 ± 2.6A
390	364 ± 7.0A	371 ± 6.0A
570	206 ± 8.7B	325 ± 1.5B
690	73 ± 14.5C	212 ± 9.5C

[a] Data represent mean values of triplicate flasks (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

sulted in mean growth of 362 ± 21.3 Klett units (mean ± standard deviation), while mean growth in medium containing all 10 of the vitamins was 361 ± 6.1 Klett units. Mineral deletion experiments indicated that magnesium was the only mineral component required for maximal growth (Table 7).

At this point, a medium containing basal salts, pantothenic acid, niacinamide, magnesium, and all 19 amino acids (MM19) supported excellent growth for 10 strains of *E. ictaluri*. Subsequent deletion studies with individual amino acids indicated that minimal amino acid requirements varied depending on the strain of *E. ictaluri* (Table 8). For purposes of defining a minimal medium, amino acids that reduced growth to less than 200 Klett units in studies with MM19 less individual amino acids (MM19-1) were used in add-back studies with the 10 individual strains. These results, reported in Table 9, indicated that the final amino acid content of the minimal medium is strain dependent. The preparation and composition of the complete defined and defined minimal media are presented in Table 1.

Growth on solid media. Colonies of *E. ictaluri* produced on BHI broth are smooth, circular, and slightly convex (7). Colony morphology on the complete defined medium and on MM19 was similar to that on BHI broth after 48 to 72 h, although the colonies on MM19 were smaller. Growth was not observed for any strain on solid minimal media with minimal amino acids.

Determination of growth curves. In general, all *E. ictaluri* strains tested reached peak cell density between 12 and 24 h of incubation in complete broth with 19 amino acids. When bacteria were grown in MM19 broth, growth and cell yields were reduced only slightly, but 48 h was required to reach peak cell density. Use of minimal medium broth containing the specific amino acids required by the individual strains resulted in detectable growth after about 48 h of incubation and peak cell density at about 96 h. For individual strains that grew well in minimal medium broth, growth was similar to that in the MM19 broth.

TABLE 3. Effect of pH on the growth of *E. ictaluri* in complete defined medium for 24 h

Initial pH	Final pH	Klett units
4.0	3.9 ± 0.006	6 ± 1.7D
5.0	4.2 ± 0.013	119 ± 3.4C
6.0	4.6 ± 0.007	298 ± 5.0B
7.0	5.1 ± 0.011	488 ± 4.7A
8.0	7.2 ± 0.095	163 ± 29.6C
9.0	8.8 ± 0.052	4 ± 2.9D

[a] Data represent mean values of triplicate flasks (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

TABLE 4. Examination of various carbon sources supporting the 24-h growth of two *E. ictaluri* strains in complete defined medium

Sugar	Klett units[a]	
	Strain 93-154	Strain 93-146
Fructose	430 ± 45.9A	478 ± 7.6A
Galactose	460 ± 25.6A	487 ± 7.5A
Glucose	472 ± 43.1A	426 ± 2.9AB
Glycerol	471 ± 17.9A	475 ± 11.1A
Mannose	274 ± 14.5B	475 ± 22.9A
Maltose	493 ± 17.5A	472 ± 10.8A
Ribose	494 ± 16.5A	499 ± 8.1A
Sucrose	241 ± 12.0B	225 ± 50.6C
None	242 ± 1.5B	351 ± 33.5B

[a] Data represent mean values of triplicate flasks of duplicated experiments (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

DISCUSSION

This report describes a complete defined medium and a minimal defined medium that support sustained growth of *E. ictaluri*. The complete defined medium, used as a base medium to develop the minimal medium, consisted of 46 individual components. The minimal medium reduces those 46 ingredients to eight essential components plus the specific amino acids required for the strain in question.

Optimal growth of *E. ictaluri* was recorded for osmolalities of 260 to 390 mosmol/kg of water, with reduced growth both above and below that range. This is in agreement with preliminary determinations in our laboratory that indicate that BHI broth, which supports excellent growth of *E. ictaluri*, has an osmolality of 360 mosmol/kg of water. Furthermore, previous work by Waltman et al. (24) and Plumb and Vinitnantharat (17) demonstrated that *E. ictaluri* is able to grow in BHI broth supplemented with up to 1.5% NaCl but not in NaCl concentrations greater than 1.5%. Plumb and Vinitnantharat (17) also showed that growth was reduced even in 0.5% NaCl compared with growth in BHI broth without NaCl. As determined in our lab, BHI broth cultures supplemented with 0.5% NaCl and 1.0% NaCl have osmolalities of 521 and 715 mosmol/kg of water, respectively.

High-density growth was observed within a narrow pH range of 7.0 to 7.5 in the defined medium. A 61% reduction in growth was observed in media at pH 6.0, with a 23 and 33% reduction in growth at pH 5.0 and 8.0, respectively. There was no growth of *E. ictaluri* in culture media at either pH 4.0 or 9.0. These data are consistent with those previously reported for BHI broth. The optimum pH range for *E. ictaluri* growth in BHI broth, as reported by Plumb and Vinitnantharat (17), was

TABLE 5. Effect of glucose concentration on the growth of *E. ictaluri* in complete defined medium

Glucose concn (g/liter)	Klett units[a]	
	12 h	24 h
0	153 ± 32.5C	192 ± 4.5D
4	426 ± 3.5A	461 ± 5.1A
20	338 ± 11.5B	420 ± 3.5B
50	97 ± 32.0C	289 ± 12.7C
100	15 ± 2.5D	15 ± 2.6E

[a] Data represent mean values of triplicate flasks (± standard deviations). Within columns, means followed by the same letter are not significantly different ($P < 0.01$).

TABLE 6. Growth of *E. ictaluri* in a defined medium deficient in a specific vitamin

Deleted vitamin	Klett units[a]
Thiamine	335 ± 17.6B
Pyridoxine	326 ± 4.5B
Pyridoxal	324 ± 5.4B
Pyridoxamine	320 ± 2.4B
Pantothenic acid	27 ± 0.82A
Riboflavin	324 ± 7.4B
Niacinamide	8 ± 1.6A
PABA	367 ± 9.5B
Biotin	327 ± 5.7B
Folic acid	335 ± 8.2B
None	361 ± 6.1B

[a] Data represent mean values of triplicate tubes at 24 h of growth (± standard deviations). Means followed by the same letter are not significantly different ($P < 0.01$).

between 7.0 and 7.5 pH units, with reduced growth at pH 6.0 and 8.0.

Although wild-type *E. ictaluri* is a facultative anaerobe, it is capable of fermenting only a limited number of carbohydrates, with only glucose, fructose, galactose, mannose, and ribose reported consistently (7, 8, 24). There are, however, variable reports concerning the ability of *E. ictaluri* to utilize glycerol. Using purple broth base, Waltman et al. (24) showed that 118 of 118 strains tested were able to grow in glycerol, while Plumb and Vinitnantharat (17), who also used purple broth base, reported that 40 of 40 strains were negative for glycerol. Our results indicate that excellent growth of *E. ictaluri* was obtained with glycerol as a carbon source. It should also be noted that *E. ictaluri* will grow weakly in defined media without sugar supplementation, presumably by using alternative carbon sources, such as amino acids, as does *Flexibacter columnaris* (22).

Variable results were obtained in this study when individual amino acids were omitted from the initial 19-amino-acid mix, with results dependent on the strain used. As indicated in Table 9, a medium composed of minimal medium supplemented with the individual amino acids that had previously given reduced growth when omitted from the various media of the MM19-1 studies did not result in a minimally defined medium for any strain examined. Absolute requirements for certain individual amino acids varied from zero to six. For example, as shown in Table 8, strains 587-671 and 89-9 appear to require only cysteine supplementation, but growth in minimal medium plus cysteine resulted in less than optimal growth of 169 Klett units for strain 587-671 and no growth for strain 89-9 (Table 9). On the other hand, strain 91-638, which also

TABLE 7. Growth of *E. ictaluri* in a defined medium deficient in a specific mineral

Deleted mineral	Klett units[a]
Magnesium	147 ± 57.0A
Iron	395 ± 49.7B
Cobalt	425 ± 49.1B
Calcium	432 ± 54.0B
Copper	432 ± 46.0B
Molybdenum	424 ± 62.9B
Manganese	433 ± 41.6B
Zinc	431 ± 41.6B
No supplement	165 ± 63.0A

[a] Data represent mean values of seven tubes at 24 h of growth (± standard deviations). Means followed by the same letter are not significantly different ($P < 0.01$).

TABLE 8. Growth of *E. ictaluri* in defined media deficient in a specific amino acid at 24 h

Deleted amino acid	Klett units of strain:									
	83-189	587-671	89-9	90-476	91-581	91-638	92-266	93-146	93-264	93-297
Alanine	488	490	450	545	387	420	337	403	350	268
Arginine	430	444	380	475	74	400	260	360	316	394
Asparagine	470	490	460	535	414	436	325	421	390	349
Aspartic acid	490	447	405	502	94	405	296	371	337	285
Cysteine	35	132	66	125	10	178	41	148	34	24
Glutamic acid	495	520	475	550	228	476	272	454	368	350
Glycine	420	380	330	455	105	349	234	344	300	190
Histidine	470	471	475	525	275	409	352	402	342	307
Isoleucine	460	447	430	523	226	428	254	400	349	354
Leucine	485	473	425	525	200	415	282	399	316	207
Lysine	480	500	495	570	318	410	232	415	345	327
Methionine	348	375	315	410	85	318	178	385	228	176
Phenylalanine	372	460	266	375	5	440	40	180	37	23
Proline	460	480	485	505	330	405	292	368	334	260
Serine	460	458	410	520	134	300	305	45	125	12
Threonine	392	414	337	470	254	364	209	325	295	268
Tryptophan	430	420	355	460	232	400	330	377	302	304
Tyrosine	405	450	345	520	235	374	111	232	300	99
Valine	465	470	470	523	167	395	186	158	325	330

appeared to require cysteine, grew well in medium without any amino acid supplementation. Some strains, such as 83-189 and 90-476, responded with excellent growth in media when only the amino acids indicated as essential in the MM19-1 studies were supplied. Strain 92-266 showed stricter amino acid requirements than did any other strain tested. This strain failed to grow when as many as nine amino acids were added back. On the other hand, the requirement for nucleotides, vitamins, and trace metals was not strain specific.

The degree of auxotrophy in *E. ictaluri* is somewhat surprising, although variable, strain-dependent amino acid or vitamin requirements are not uncommon in bacteria (4, 12, 25). *E. ictaluri* is reported to be extremely stable and homogeneous in its biochemical reactivity (24), plasmid profile (10, 14, 23), and serological relatedness (1, 16, 17). Recent results indicate that *E. ictaluri* possesses one or two restriction endonucleases that

TABLE 9. Effect of supplementation with various amino acids on the growth of 10 strains of *E. ictaluri* in a defined minimal medium

Supplemented amino acid(s)[a]	Klett units of strain[b]:									
	83-189	587-671	89-9	90-476	91-581	91-638	92-266	93-146	93-264	93-297
None						439		147	93	102
C	347	169	NG[c]	355	NG	370	NG	219	170	140
S						395		136	113	123
C, F	268	107	31	250	NG	378	NG	260	223	170
C, F, S						240		205	128	98
C, F, V	303	33	NG	144	NG		NG			
C, F, S, V						262		192	171	103
C, F, V, M, Y	260	150	NG	244	NG		NG			
C, F, V, M, Y, L, P, S, D	404	390	390	395	230		NG			
C, F, S, M, Y, G						335		200	159	210

[a] C, cysteine; S, serine; F, phenylalanine; V, valine; M, methionine; Y, tyrosine; L, leucine; P, proline; D, aspartic acid; G, glycine.

[b] Values at 96 h of growth are given. Combinations where no values are given were not evaluated.

[c] NG, no growth.

TABLE 1. Preparation and composition of defined[a] and defined minimal[b] media for *E. ictaluri*

Ingredient	Stock solution concn	Final medium concn [mg/liter (mM)]
M9 salts		
$Na_2HPO_4 \cdot 7H_2O$	60 g/liter	15,000 (56)
KH_2PO_4	30 g/liter	7,500 (55)
NH_4Cl	10 g/liter	2,500 (47)
NaCl	5 g/liter	1,250 (21)
Glucose	200 g/liter	4,000 (22)
$MgSO_4 \cdot 7H_2O$	240 g/liter	240 (0.970)
$FeSO_4 \cdot 7H_2O$	5.0 g/liter	5 (0.018)
Minerals		
$MnSO_4 \cdot H_2O$	1.0 g/liter	5 (0.029)
$CaCl_2 \cdot 2H_2O$	3.0 g/liter	15 (0.012)
$ZnSO_4 \cdot 7H_2O$	1.0 g/liter	5 (0.017)
$CuSO_4 \cdot 5H_2O$	0.02 g/liter	0.1 (4.0×10^{-4})
$CoCl_2 \cdot 6H_2O$	0.02 g/liter	0.1 (4.0×10^{-4})
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02 g/liter	0.1 (8.0×10^{-5})
Vitamins		
p-Aminobenzoic acid	0.04 g/liter	0.1 (7.3×10^{-4})
Niacinamide	0.4 g/liter	1.0 (8.2×10^{-3})
DL-Pantothenic acid, calcium salt	0.2 g/liter	0.5 (2.1×10^{-3})
Pyridoxal HCl	0.12 g/liter	0.3 (1.5×10^{-3})
Pyridoxamine $(HCl)_2$	0.12 g/liter	0.3 (1.2×10^{-3})
Pyridoxine HCl	0.4 g/liter	1.0 (4.9×10^{-3})
Riboflavin	0.2 g/liter	0.5 (1.3×10^{-3})
Thiamine HCl	0.2	0.5 (1.5×10^{-3})
d-Biotin	—[c]	0.001 (4.1×10^{-6})
Folic acid	—	0.01 (2.3×10^{-5})
Purines and pyrimidines[d]		
Adenine sulfate $\cdot H_2O$	1.0 g/liter	10 (0.054)
Uracil	1.0 g/liter	10 (0.089)
Guanine HCl $\cdot H_2O$	1.0 g/liter	10 (0.054)
Xanthine	1.0 g/liter	10 (0.066)
Amino acids[e]		
L-Alanine	400 mg (dry wt)	200 (2.2)
L-Arginine	484 mg (dry wt)	242 (1.4)
L-Asparagine	800 mg (dry wt)	400 (3.0)
L-Aspartic acid	200 mg (dry wt)	100 (0.75)
L-Cysteine	100 mg (dry wt)	50 (0.41)
L-Glutamic acid	600 mg (dry wt)	300 (2.0)
Glycine	200 mg (dry wt)	100 (1.3)
L-Histidine	124 mg (dry wt)	62 (0.39)
L-Isoleucine	500 mg (dry wt)	250 (1.9)
L-Leucine	500 mg (dry wt)	250 (1.9)
L-Lysine	500 mg (dry wt)	250 (1.4)
DL-Methionine	200 mg (dry wt)	100 (0.67)
L-Phenylalanine	200 mg (dry wt)	100 (0.61)
L-Proline	200 mg (dry wt)	100 (0.87)
L-Serine	100 mg (dry wt)	50 (0.48)
L-Threonine	400 mg (dry wt)	200 (1.7)
L-Tryptophan	80 mg (dry wt)	40 (0.20)
L-Tyrosine	200 mg (dry wt)	100 (0.55)
L-Valine	500 mg (dry wt)	250 (2.1)

[a] Defined medium was made by combining 250 ml of M9 salts, 20 ml of glucose solution, 1 ml of $MgSO_4$ solution, 1 ml of $FeSO_4$ solution, 5 ml of trace minerals, 20 ml of each purine and pyrimidine, 3 ml of vitamins, and 3.1 g of the amino acid mixture dissolved in 500 ml of distilled water. The volume was brought to 1 liter with warm distilled water, and the pH was adjusted to 7.0. The medium was filter sterilized with a 0.45-μm-pore-size filter.

[b] Defined minimal medium was made by combining 250 ml of M9 salts, 20 ml of glucose solution, 1 ml of $MgSO_4$ solution, 3 ml of vitamins containing only niacinamide and pantothenic acid, and the appropriate amino acids for the strain dissolved in 500 ml of distilled water. The volume was brought to 1 liter with warm distilled water, and the pH was adjusted to 7.0. The medium was filter sterilized with a 0.45-μm-pore-size filter.

[c] —, biotin and folic acid were prepared in concentrations of 1 mg/ml. Biotin was solubilized by warming, and folic acid was solubilized by the addition of NH_4OH. From these solutions, 0.02 ml of biotin and 0.2 ml of folic acid were added to a 50-ml solution of the remaining vitamins, brought to a final volume of 60 ml, and filter sterilized.

[d] Separate xanthine and guanine stock solutions of 1 mg/ml were made by adding concentrated HCl drop by drop until dissolved. Adenine and uracil were dissolved separately in warm water to the same concentration.

[e] Amino acids were combined dry in the indicated amounts, ground in a mortar and pestle, and stored at 20°C. A total of 3.1 g of the amino acid mixture was added per 1,000 ml of medium.

plete defined medium (pH 7.0) with concentrations ranging from 120 to 700 mosmol/kg of H_2O. Osmolality was varied by changing the concentration of the M9 salt solution and confirmed with a model 3DII DiGi-Matic osmometer (Advanced Instruments, Needham Heights, Mass.).

Carbon source utilization was determined for fructose, glucose, galactose, glycerol, maltose, mannose, ribose, and sucrose at a concentration of 4 g/liter. Although all sources except sucrose provided for saturated growth of *E. ictaluri*, glucose was selected for determination of optimal concentration by inoculating complete defined medium at pH 7 containing concentrations of 0, 4, 20, 50, and 100 g/liter.

Optimal pH was determined by dividing a batch of complete defined medium into six aliquots and adjusting the pH to 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 by adding either 1 N NaOH or 1 N HCl. The pH of the medium was measured prior to inoculation (initial) and after 24 h of incubation (final).

The optimized complete defined medium was then used in a series of deletion and add-back experiments to determine the minimal essential nutrients required for growth of *E. ictaluri*. Initial deletion of entire nutrient groups, including 10 vitamins, eight minerals, two purines and two pyrimidines, or 19 amino acids, was followed by deletions of individual components when the group was determined to be necessary for growth. Subsequently, each nutrient that yielded no growth when deleted was individually restored to the medium to confirm that it was essential for growth.

Determination of growth curves. Growth of *E. ictaluri* in complete broth medium with 19 amino acids was compared with growth in minimal medium with 19 amino acids and with growth in minimal medium containing only the specific amino acids required for growth of each particular strain (see below). Cultures were grown in 250-ml Klett flasks at 30°C, and cell density was measured in Klett units for a 144-h time period. Samples for the first 24 h were taken at 2-h intervals, and subsequent samples were taken every 12 h.

Statistical analysis. Data were analyzed by analysis of variance. For effects found to be significant, paired comparisons were evaluated by Tukey's procedure (Statistix Version 4.1; Analytical Software, Tallahassee, Fla.).

RESULTS

Optimal osmolality for growth was observed at 260 and at 390 mosmol/kg (Table 2). An osmotic concentration of 570 mosmol/kg resulted in a slight reduction in bacterial growth, while growth was suboptimum at concentrations of 120 and 690 mosmol/kg. Although bacterial growth levels in salt concentrations of 260 and 390 mosmol/kg were equivalent, 390 mosmol/kg was selected for subsequent experiments. Optimal pH for growth was observed at pH 7.0 (Table 3). Subsequent testing of a variety of carbon sources, including fructose, galactose, glucose, glycerol, maltose, mannose, ribose, and sucrose, indicated that there was little significant difference in growth between many of them after 24 h (Table 4). Glucose was selected for determination of optimal concentration by evaluating *E. ictaluri* cultures grown with various glucose concentrations, and because growth in 4 g of glucose per liter was significantly higher at 12 and 24 h of incubation than any other glucose concentration, the concentration of 4 g/liter was chosen for subsequent experiments (Table 5).

Initial determination of vitamin, purine and pyrimidine, mineral, and amino acid requirements utilized a defined medium that was made deficient in the individual nutrient groups. Results indicated that deletion of the nucleotides did not affect growth, deletion of the vitamins or amino acids completely abrogated growth, and deletion of the minerals reduced final Klett readings by about 50%.

Subsequent deletion of individual vitamins indicated that pantothenic acid and niacinamide were essential for growth (Table 6). Testing of four strains in triplicate tubes of defined medium containing only pantothenic acid and niacinamide re-

may act to restrict the intrusion of foreign DNA (26). This could explain the homogeneity of the major phenotypic traits and also account for the persistence of minor phenotypic mutations.

The possible toxic effects of various amino acids in *E. ictaluri* are not clear, although some amino acid toxicity was observed with *E. ictaluri* when essential amino acids were reintroduced in various combinations. For example, when valine was added in combination with cysteine and phenylalanine at concentrations described in Table 1, a threefold drop in growth occurred in the cysteine-requiring strains. However, when a number of other amino acids, such as methionine and tyrosine or methionine, tyrosine, leucine, proline, or serine, were added to valine, cysteine, and phenylalanine, the growth level was restored to that obtained with cysteine and phenylalanine alone. Further work is required to determine the synergistic or antagonistic effects of the amino acid combinations utilized by various strains of *E. ictaluri*.

Combination toxicity for amino acids was first observed by Gladstone (5) for *Bacillus anthracis*. The amino acids that abrogate these toxic effects were themselves toxic when added singly. The author also showed that certain amino acids are necessary for growth but not essential since growth still occurs, although at a lower rate, when these amino acids are absent. Reduction of growth rate may indicate that the organism can synthesize some amino acids when they are not added to the medium (5).

Culture of *E. ictaluri* generally requires the use of complex or enriched media for growth. Although a medium for the selective isolation of *E. ictaluri* that is useful in the isolation of *E. ictaluri* from mixed flora in environmental reservoirs and clinical sources was described previously (21), it is also of a complex, undefined formulation. The minimal medium reported here is well defined and should be preferable to complex media for conducting future physiological, nutritional, and pathogenicity studies on *E. ictaluri*.

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